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Apolipoprotein B Determination in the Dissolved Precipitate Obtained after Precipitation of LDL with Polyvinylsulphate

An Alternative Method for the Determination of LDL Apolipoprotein B without Using Ultracentrifugation

By *H. Schriewer, W. Nolte*

Institut für Klinische Chemie und Laboratoriumsmedizin (Zentrallaboratorium) der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität Münster

H. Robenek

Lehrstuhl für Medizinische Cytobiologie der Westfälischen Wilhelms-Universität Münster and

G. Assmann

Institut für Klinische Chemie und Laboratoriumsmedizin (Zentrallaboratorium) der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität Münster

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Summary: Using a commercially available test for LDL cholesterol (Boehringer Mannheim), a method was developed for determination of LDL apolipoprotein B without using ultracentrifugation. The infranatant obtained by precipitation of serum with polyvinylsulphate was redissolved in a saline citrate solution and incubated with phospholipase A_2, phospholipase C, phospholipase D or triglyceride lipase, respectively. When the saline-citrate redissolved precipitate was monitored by electron microscopy, it appeared as a fibriform network. The additional incubation with phospholipase A_2, C, D, or triglyceride lipase resulted in a molecularization of LDL particles. By electron microscopy these particles could not be distinguished from LDL particles isolated by ultracentrifugation. In radial immunodiffusion tests, the additional incubation of the redissolved precipitate with phospholipase C or phospholipase D resulted in a total loss of the slight immunoreactivity observed before phospholipase incubation. However, additional incubation with triglyceride lipase resulted in a significant increase in immunoreactivity. Only the additional incubation of the redissolved precipitate with phospholipase A_2 resulted in an immunoprecipitation reaction comparable to that with LDL particles isolated by ultracentrifugation. Using a resolubilized and phospholipase A_2-incubated precipitate of a pool serum as apolipoprotein B standard, a good correlation was obtained between apolipoprotein B values measured in this dissolved precipitate and those measured in the $d > 1.006$ kg/l fraction isolated by ultracentrifugation ($r = 0.95$; $y = 0.95x + 0.018$; $n = 44$).

Apolipoprotein B-Bestimmung im Präzipitat nach Fällung von LDL mit Polyvinylsulfat
Eine alternative Methode zur Bestimmung von LDL-Apolipoprotein B ohne Ultrazentrifugation

Zusammenfassung: Auf der Basis eines kommerziell erhältlichen Tests zur LDL-Cholesterinbestimmung (Boehringer-Mannheim) wurde eine Methode zur Bestimmung von LDL-Apolipoprotein B ohne Ultrazentrifugation entwickelt.

Das nach Fällung von Seren mit Polyvinylsulfat auftretende Präzipitat wurde in Natriumchlorid-Citratlösung aufgelöst und in Gegenwart von Phospholipase A_2, Phospholipase C, Phospholipase D oder Triglyceridlipase inkubiert. Das mit Natriumchlorid-Citratlösung aufgelöste Präzipitat zeigte elektronenmikroskopisch ein fibriformes Netzwerk. Die zusätzliche Inkubation mit Phospholipase A_2, C, D oder Triglyceridlipase bewirkte eine Rekonstitution der LDL-Partikel. Elektronenoptisch waren diese Partikel nicht von LDL-Partikeln zu unterscheiden, die durch Ultrazentrifugation isoliert wurden. Die zusätzliche Inkubation der aufgelösten Präzipitate mit Phospholipase C oder Phospholipase D führte zu einem vollständigen Verlust der Immunreaktivität bei Durchführung der radialen Immundiffusion, während die zusätzliche Inkubation mit Triglyceridlipase eine leichte Steigerung der Immunreaktivität ergab. Die zusätzliche Inkubation der aufgelösten Präzipitate mit Phospholipase A_2 bewirkte dagegen eine Immunpräzipitation, die derjenigen von durch Ultrazentrifugation isolierten LDL-Partikeln entsprach.

Der Vergleich der Apolipoprotein B-Werte im aufgelösten Präzipitat mit denen der durch Ultrazentrifugation isolierten $d > 1{,}006$ kg/l Fraktion ergab eine Übereinstimmung der Wertepaare, wenn als Standard ein aufgelöstes und Phospholipase A_2-behandeltes Polyvinylsulfat-Präzipitat eines Poolserums verwandt wurde ($r = 0{,}95$, $y = 0{,}95x + 0{,}018$, $n = 44$).

Introduction

Previous epidemiological studies have shown that an elevated concentration of LDL cholesterol represents a risk factor for coronary heart disease (1—2). But LDL are not homogeneous; rather, they comprise a relatively heterogeneous group of particles of which apolipoprotein B, in particular, is the marker by which peripheral and hepatic receptors recognize LDL particles (3). Because of the key role of apolipoprotein B in LDL metabolism it may be, theoretically, a better predictor of coronary heart disease than LDL cholesterol (4). The accurate determination of LDL apolipoprotein B, however, implies a time-consuming isolation of LDL by ultracentrifugation. Recently, *Sniderman* et al. (4) have measured apolipoprotein B levels in LDL separated from VLDL by exclusive gel radial immunodiffusion. However, the accuracy of this method may be limited because small VLDL particles may interfere with the determination of LDL apolipoprotein B.

In the present paper we describe a new method for determination of LDL apolipoprotein B. The method is based on the selective precipitation of LDL from whole serum with polyvinylsulphate (5), using a recently developed commercial test for the analysis of LDL cholesterol.

Materials and Methods

Materials

Reagents

LDL cholesterol precipitant reagent, containing 1 g/l polyvinylsulphate, 169 g/l polyethyleneglycol methylether and 5 mmol/l Na_2 EDTA (catalogue No. 726290), phospholipase A_2 (EC 3.1.1.4 (6000 kU/l), order No. 161454), phospholipase C (EC 3.1.4.3 (1600 kU/l), order No. 108502), phospholipase D (EC 3.1.4.4 (50 kU/l), order No. 108537), and "test combination Triglyceride vollenzymatisch UV Test" (order no. 240052) were purchased from Boehringer Mannheim, D-6800 Mannheim 31, FRG.

Immunoscientific Anti-apo B (order No. 4834005) was obtained from Immuno AG, Vienna, Austria, apolipoprotein B standard serum (order No. OTRF 02/03) from Behringwerke AG, D-3550 Marburg, FRG.

Servablue R was obtained from Serva, D-6900 Heidelberg, FRG, and triglyceride lipase (1.0 kU/ml, order No. 437611) from Calbiochem, Behring Corp., La Jolla, CA 92037, USA.

Tris-barbital-lactate buffer, pH 8.6, contained 0.366 mol/l Tris, 0.120 mol/l barbituric acid, 15.2 mmol/l Na azide, 1.68 mmol/l Ca lactate. For radial immunodiffusion assay this buffer was diluted 1 : 8 with distilled water.

Thesit (dodecyl alcohol polyoxyethylene ether) was obtained from Desitin-Werke, C. Klinke GmbH, D-2000 Hamburg, FRG, Apovax from Ortho Diagnostic, Belgium and Triton X-100 from Sigma Chemie GmbH, D-8028 Taufkirchen, FRG.

Sample material

As test material we used fresh serum from the test series "Prospective epidemiological study in company employees in Westphalia" (6).

Procedures

Precipitation of LDL with polyvinylsulphate

Precipitation of LDL with polyvinylsulphate was performed as previously described (5). For the LDL precipitation 100 µl serum were mixed in an incubation tube (Sarsted No. 52690) with 50 µl precipitation reagent, left at room temperature for 15 minutes and centrifuged in an Eppendorf Centrifuge 3200 for 2 × 2 minutes. The precipitate obtained by careful and complete decantation of the supernatant was resolubilized with 600 µl of a solution containing 0.15 mol/l trisodium citrate and 0.11 mol/l sodium chloride and mixed thoroughly with a vortex for 2 × 4 minutes.

Incubation of resolubilized polyvinylsulphate-induced precipitate with lipases

Dissolved precipitate (100 µl) was thoroughly mixed with 6 µl phospholipase A_2 or 10 µl phospholipase C, or 100 µl phospholipase D, or 100 µl triglyceride lipase, respectively (7). The mixture was incubated at 40 °C (phospholipase A_2) or 37 °C (phospholipase C, phospholipase D, triglyceride lipase) for 6–24 hours, followed by determination of apolipoprotein B.

Determination of apolipoprotein B by radial immunodiffusion

RID was performed according to *Mancini* et al. (8). Anti-apolipoprotein-B (40 µl) was mixed with 20 ml of 1% solution of agarose in Tris-barbital-lactate buffer pH 8.6. The volume of the sample was 5 µl. Apolipoprotein B standard serum was diluted with 0.15 mol/l NaCl 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32. Incubation time was 4 days at room temperature. The diameters of the precipitation rings obtained were measured following staining with Servablue R.

Preparative isolation of lipoproteins by ultracentrifugation

Preparative isolation of lipoproteins by ultracentrifugation was performed as previously described (9).

Negative staining and electron microscopy

Negative staining for electron microscopy was performed by placing a formvar-coated grid for 5 minutes on a drop of diluted solution of lipoproteins, which was then transferred to a drop of distilled water for 1 minute and finally to a drop of 20 g/l uranyl acetate, pH 3.5, for 5 minutes. The grids were examined in a Philips EM 201 operated at 60 kV.

Results

Influence of phospholipases and detergents on the constitution of LDL particles in the precipitate obtained after addition of polyvinylsulphate to serum

Figure 1 shows an electron micrograph of the d 1.006–1.063 kg/l fraction isolated by ultracentrifugation. The fraction consists of homogeneous and monodispersed particles with a mean diameter of 20.7 nm (fig. 1a). In contrast to the d 1.006–1.063 kg/l fraction, the electron microscopy of a precipitate dissolved in citrate saline solution shows an heterogeneous and fibrillar network lacking monodispersed particles (fig. 1b). The addition of each of the detergents Thesit (1.0%), Triton X-100 (1.0%) and Apovax (1.0%) did not result in a reconstitution of lipoprotein particles visible by electron microscopy. However, incubation of the precipitate with phospholipase A_2 (340 kU/l final concentration) for more than 6 hours at 40 °C resulted in a reconstitution of lipoprotein particles (fig. 1c). The phospholipase A_2-treated precipitate consists of monodispersed and homogeneous particles with a mean diameter of 20.8 nm. By electron microscopy these lipoprotein particles were indistinguishable from particles obtained by ultracentrifugation. Similar results were obtained by using triglyceride lipase (500 kU/l final concentration) or other lipases like phospholipase C (145.5 kU/l final concentration) and phospholipase D (25 kU/l final concentration).

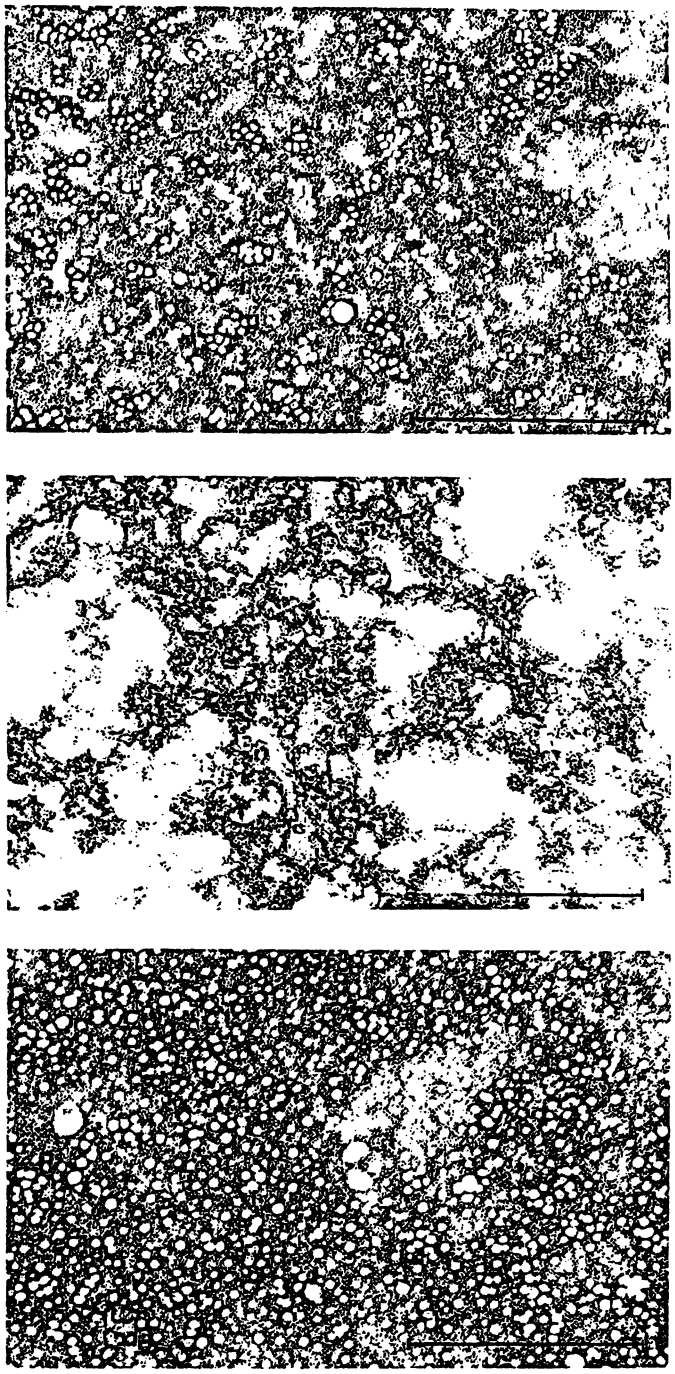

Fig. 1. a) Electron micrograph of the d 1.006–1.063 kg/l fraction isolated by ultracentrifugation.
b) Electron micrograph of the polyvinylsulphate-induced precipitate dissolved in citrate saline solution.
c) Electron micrograph of the redissolved polyvinylsulphate-induced precipitate incubated with phospholipase A_2 (340 kU/l final concentration) for 6–24 h at 40 °C. Bar: 9.5 nm.

Influence of phospholipases and triglyceride lipase on the diameter of the precipitation rings using radial immunodiffusion (RID)

In the RID only small precipitation rings were obtained when the precipitate dissolved in citrate saline solution was used as the sample (fig. 2). The additional incubation of the dissolved precipitate with phospholipase C (145.5 kU/l final concentration) or phospholipase D (25 kU/l final concentration) eliminated any visible immunoprecipitation. The additional incubation of the dissolved precipitate with triglyceride lipase (500 kU/l final concentration), however, resulted in enhanced immunoprecipitation. A further increase of added triglyceride lipase > 500 kU/l final concentration caused no enhancement of the diameter of the precipitation rings. Incubation of the dissolved precipitate with phospholipase A_2 (340 kU/l final concentration) resulted in a major enhancement of the immunoreaction exceeding that of lipase incubation. Optimal conditions were found when phospholipase A_2 was incubated at a final concentration of 340 kU/l for 6 h at 40 °C.

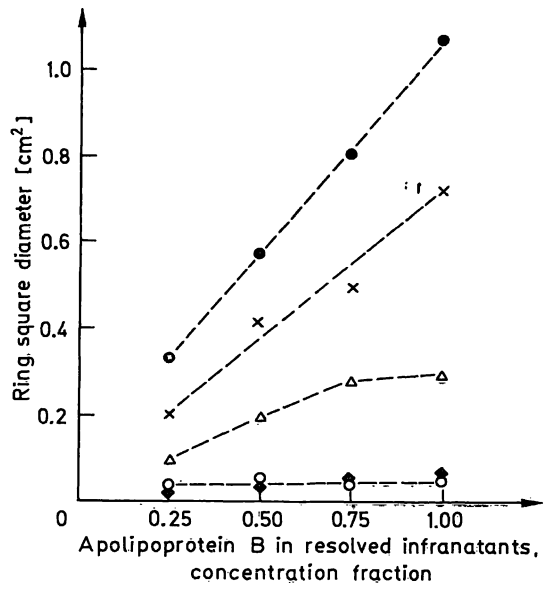

Fig. 2. Influence of the additional incubation of the dissolved polyvinylsulphate-induced precipitate with phospholipase A_2 (340 kU/l), phospholipase C (145.5 kU/l), phospholipase D (25 kU/l) and triglyceride lipase (500 kU/l) on the square diameters of the precipitation rings using radial immunodiffusion.
●—● phospholipase A_2
○—○ phospholipase C
△—△ no addition of enzymes
x—x triglyceride lipase
◆—◆ phospholipase D.

Influence of freezing on the immunoprecipitation

When dissolved and phospholipase A_2-incubated precipitates were stored for 6 months at −20 °C, identical values were obtained when compared with dissolved and phospholipase A_2-incubated precipitates from the fresh samples.

Precision of apolipoprotein B determination of the dissolved polyvinylsulphate induced precipitates

With regard to the precision in series, a variation coefficient of 6% (n = 15; $\bar{x}$ = 1.0 g/l) was obtained. Day to day precision (8%; n = 15; $\bar{x}$ = 0.95 g/l) was tested with samples of frozen pool serum.

Accuracy of apolipoprotein B determination in dissolved precipitates

The accuracy of the apolipoprotein B measurements in dissolved precipitates was determined by comparison with corresponding apolipoprotein B values measured in the d > 1.006 kg/l fraction isolated by ultracentrifugation. As the standard for apolipoprotein B determination in dissolved precipitates, we used a resolubilized and phospholipase A_2-incubated precipitate of a pool serum. The apolipoprotein B concentration of this tertiary standard was determined by RID as apolipoprotein B in the d > 1.006 kg/l fraction using a commercially available apolipoprotein B standard serum (Behring Werke AG) as reference material. Comparison of the apolipoprotein B values measured in the dissolved precipitate of normo- and hypertriglyceridaemic sera (0.46—8.55 mmol/l triglycerides) with the corresponding apolipoprotein B values measured in the d > 1.006 kg/l fraction, showed a good correlation (r = 0.95; y = 0.95x + 0.018; n = 44) (fig. 3). The mean value obtained from the dissolved precipitate ($\bar{y}$ = 0.607) was nearly identical to the mean value obtained by the reference method ($\bar{x}$ = 0.616).

Discussion

In previous studies we have shown that the addition of polyvinylsulphate to serum in different concentrations resulted in a complete precipitation of LDL and Lp(a) without co-precipitation of VLDL and HDL (5). The simultaneous addition of polyethyleneglycol methylether is necessary, in order to obtain complete and selective precipitation of LDL with polyvinylsulphate. Polyethyleneglycol methylether is supposed to remove the hydration layer of LDL, so that binding of polyvinylsulphate to LDL particles is strengthened (*Ziegenhorn, J. & Draeger, B.*; unpublished). The

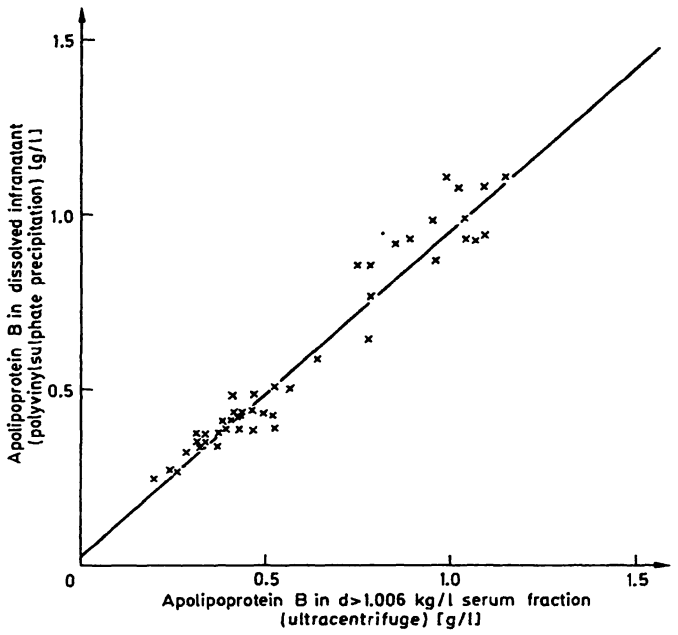

Fig. 3. Regression analysis of apolipoprotein B values measured in the phospholipase A_2-incubated polyvinylsulphate-induced precipitate (y) and apolipoprotein B values measured in the d > 1.006 kg/l ultracentrifugation fraction (x) of normo- and hypertriglyceridaemic sera (0.46–8.45 mmol/l triglycerides).
r = 0.95; y = 0.95x + 0.018; n = 44.

polyvinylsulphate precipitation method described is in principle a further development of former precipitation techniques, which used polysaccharides such as amylopectin or dextran sulphate (10). These previous studies have shown that complete precipitation and reproducibility of results depend on the degree of polymerisation and the sulphate content per monomer of the polysaccharides used. In synthetic structures, a defined sulphate content per monomer, as well as a constant degree of polymerisation, can be obtained.

The sulphate groups of polyvinylsulphate are supposed to aggregate with the positively-charged basic LDL particles (*Ziegenhorn, J. & Draeger, B.*, unpublished). Furthermore, it has been supposed that in addition to ionogenic binding, pH-independent hydrophobic interactions are involved. As shown by electron microscopy, the precipitation of LDL by polyvinylsulphate resulted in a masking of the typical structure of LDL particles. However, the original structure of monodispersed LDL particles does not seem to be destroyed by polyvinylsulphate precipitation. The cleavage of phospholipids by phospholipase A_2, C or D respectively, as well as the addition of triglyceride lipase resulted in the reappearance of lipoprotein particles which could not be distinguished from native LDL particles isolated by ultracentrifugation. As shown by *Nishida* (11), phospholipase A treatment of insoluble complexes obtained after addition of dextran sulphate to LDL also resulted in the production of soluble and deaggregated LDL complexes. This result suggests that fatty acids caused the formation of soluble complexes by the association of negatively charged fatty acids with the lipoproteins. Possibly, charge interactions are of importance in the resolution of insoluble LDL polyanion complexes.

It has been shown (12) that the incubation of LDL particles isolated by ultracentrifugation with phospholipase A_2 does not affect the composition or the electron microscopic structure of the LDL particles. Furthermore, the immunoreactivity of LDL is not influenced by the phospholipase A_2 treatment of LDL. In our experiments the addition of phospholipase A_2 resulted in an increase of immunoreactivity of apolipoprotein B in dissolved polyvinylsulphate, as compared to untreated, dissolved precipitates. Apparently, the increase in the immunoreactivity of the phospholipase A_2-treated, redissolved precipitates reflects a "normalization" of immunoreactivity of particle-bound apolipoprotein B. However, the cleavage of the phosphorylcholine or choline components of choline-containing phospholipids resulted in a total disappearance of the immunoreactivity of lipoprotein-bound apolipoprotein B. Possibly, the phospholipase C and phospholipase D treatment altered the conformation of apolipoprotein B, so that the immunoreaction was inhibited.

Our results show that the method described can be used for routine determination of LDL apolipoprotein B. However, LDL fractions isolated by ultracentrifugation cannot be used as standards, since there is a difference in the immunoreactivity between LDL fractions isolated by ultracentrifugation and those isolated with polyvinylsulphate. The problem can be solved by using phospholipase A_2-treated, dissolved precipitate as a standard, and determination of apolipoprotein B in the d> 1006 kg/l fraction of the same serum. According to our results, phospholipase A_2-incubated dissolved precipitate can be refrigerated with no change in immunoreactivity. The coprecipitation of Lp(a) may be a disadvantage for the accurate determination of LDL apolipoprotein B using the method described. In sera containing Lp(a) values < 0.10 g/l (total Lp(a) mass measured by rocket electrophoresis (13)), Lp(a) apolipoprotein B does not have to be taken into account. In sera containing Lp(a) concentrations > 0.10 g/l, the LDL apolipoprotein B values should be calculated by appropriate corrections (Lp(a) contains approximately 18% apolipoprotein B related to total Lp(a) mass) (14).

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Professor Dr. H. Schriewer
Institut für Klinische Chemie
und Laboratoriumsmedizin
Medizinische Einrichtungen
der Westf. Wilhelms-Universität
Albert-Schweitzer-Straße 33
D-4400 Münster